

## Defining the Transfer Functions of the PCAD Model in North Atlantic Right Whales (*Eubalaena glacialis*) – Retrospective Analyses of Existing Data

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Award Number: N000141010614

<http://tinyurl.com/RightWhaleConservationMedicine>

<http://tinyurl.com/MarineStress>

### LONG-TERM GOALS

Anthropogenic noise is known to cause both behavioral and physiological changes in marine mammals, but the potential for long-term population level effects is not known. The NRC (2005) Population Consequences of Acoustic Disruption (PCAD) model provided a framework to trace the effects of acoustic disturbance through the life history of a marine mammal to its population status. In North Atlantic right whales (*Eubalaena glacialis*), extensive data on hormone levels, health and body condition, and individual life history exists. Our long term goal was to analyze the links between hormones, visual assessments of health, and vital rates of right whales, enhance the modeling efforts on PCAD transfer functions and develop a theoretical framework for field studies on acoustic disturbance.

### OBJECTIVES

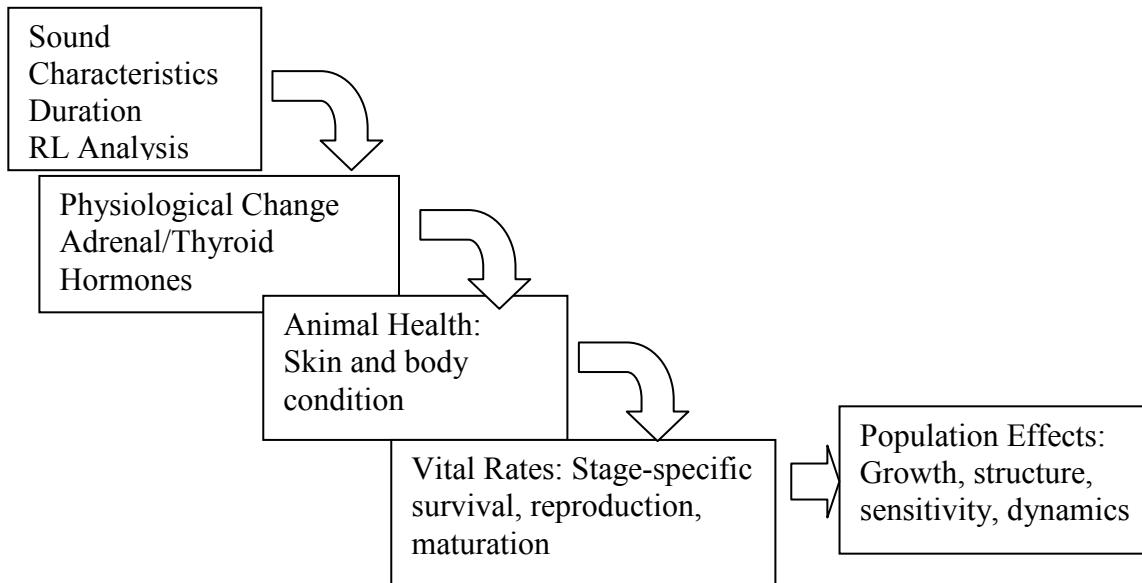
The objectives were to test an alternative approach to elements of the PCAD model by: 1) substituting “behavior change” with direct measurements of physiological changes (using fecal hormone levels - Hunt et al. 2006; Rolland et al. 2005); 2) replacing “life function” with skin and body condition indices (Pettis et al. 2004), and 3) investigating the links between these parameters and right whale survival, reproduction and maturation (Fig. 1). Analyses of retrospective data and new data on fecal thyroid hormones (in FY 2012) combined with updated health indices are now available to assess the effects of a variety of disturbances in right whales.

### APPROACH

The approach over the last three years has been to develop models of the relationships between fecal stress and reproductive hormone levels to characterize (and control for) variations in stress hormones that occur with different sexes, ages and reproductive states; model the links between body and skin condition scores and fecal stress hormones; analyze the hormone and acoustic data collected before and after 9/11 to evaluate the effects of underwater noise on whale physiology; conduct retrospective hormone assays; and develop new hormone assays. Our team included Scott Kraus, New England Aquarium (right whale biology), Roz Rolland, New England Aquarium (mammalian physiology,

<b>Report Documentation Page</b>			Form Approved OMB No. 0704-0188	
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1. REPORT DATE <b>2012</b>	2. REPORT TYPE <b>N/A</b>	3. DATES COVERED <b>-</b>		
4. TITLE AND SUBTITLE <b>Defining the Transfer Functions of the PCAD Model in North Atlantic Right Whales (<i>Eubalaena glacialis</i>) Retrospective Analyses of Existing Data</b>			5a. CONTRACT NUMBER	
			5b. GRANT NUMBER	
			5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)			5d. PROJECT NUMBER	
			5e. TASK NUMBER	
			5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) <b>New England Aquarium Central Wharf Boston, MA 02110</b>			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)			10. SPONSOR/MONITOR'S ACRONYM(S)	
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT <b>Approved for public release, distribution unlimited</b>				
13. SUPPLEMENTARY NOTES <b>The original document contains color images.</b>				
14. ABSTRACT				
15. SUBJECT TERMS				
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT <b>SAR</b>	18. NUMBER OF PAGES <b>12</b>
a. REPORT <b>unclassified</b>	b. ABSTRACT <b>unclassified</b>	c. THIS PAGE <b>unclassified</b>		19a. NAME OF RESPONSIBLE PERSON

health assessments), Peter Corkeron, National Marine Fisheries Service (statistics), Kathleen Hunt (endocrinology) and Susan Parks, The Pennsylvania State University (acoustics).



**Figure 1.** Right whales and acoustic disturbance – the proposed adaptation of the PCAD model. In this alternative framework, we substituted “behavioral changes” resulting from acoustic disturbance with “physiological changes” using adrenal and thyroid hormones, and the “life function changes” with “animal health changes” using visual assessment of body and skin condition.

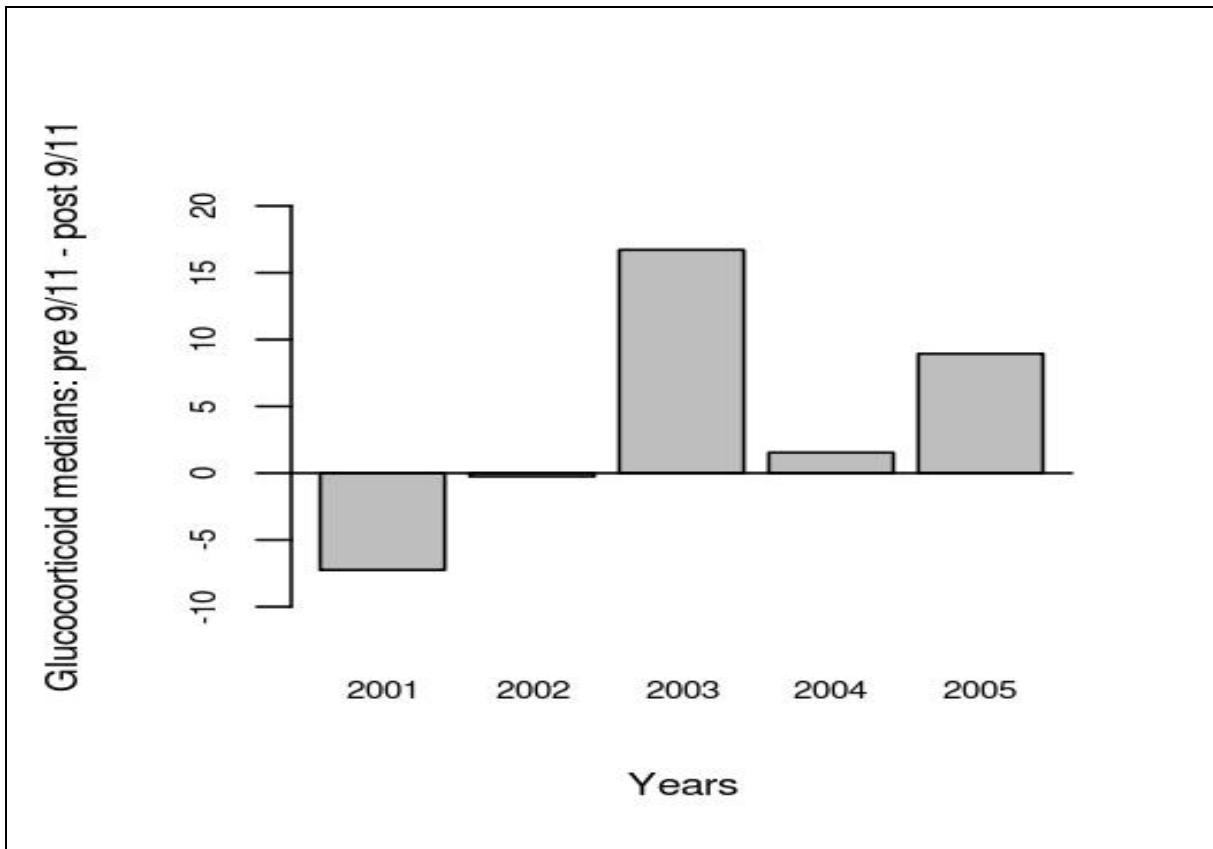
## WORK COMPLETED

All proposed work has been completed. Although our work was organized by tasks, this final report presents the findings on first three transitions in the alternative PCAD framework (Fig. 1).

### TRANSITION 1: ACOUSTIC DISTURBANCE IS LINKED TO PHYSIOLOGICAL AND HORMONAL CHANGES

#### ***Evidence that Increased Underwater Noise Elevates Whale Stress Hormones***

The events of 11 September 2001 (9/11) led to a 6dB decrease in underwater noise, with more significant reductions below 150 Hz related to reduced ship traffic following 9/11 in the Bay of Fundy. We measured levels of glucocorticoid (GC) metabolites (“stress hormones”) in right whale fecal samples before ( $n = 114$ ) and after 11 September ( $n = 30$ ) for the years 2001-2005. The only year in which there was a significant decrease in fecal GCs after 11 September was 2001 with a significant effect of year and period (Kruskall-Wallis  $X^2 = 29.6889$ , df = 4,  $P < 0.0001$ ) (Fig. 2). This is the first evidence that exposure to underwater noise from large ships is associated with elevated stress in baleen whales, which has implications for whale populations in heavy ship traffic areas (Rolland et al. 2012).

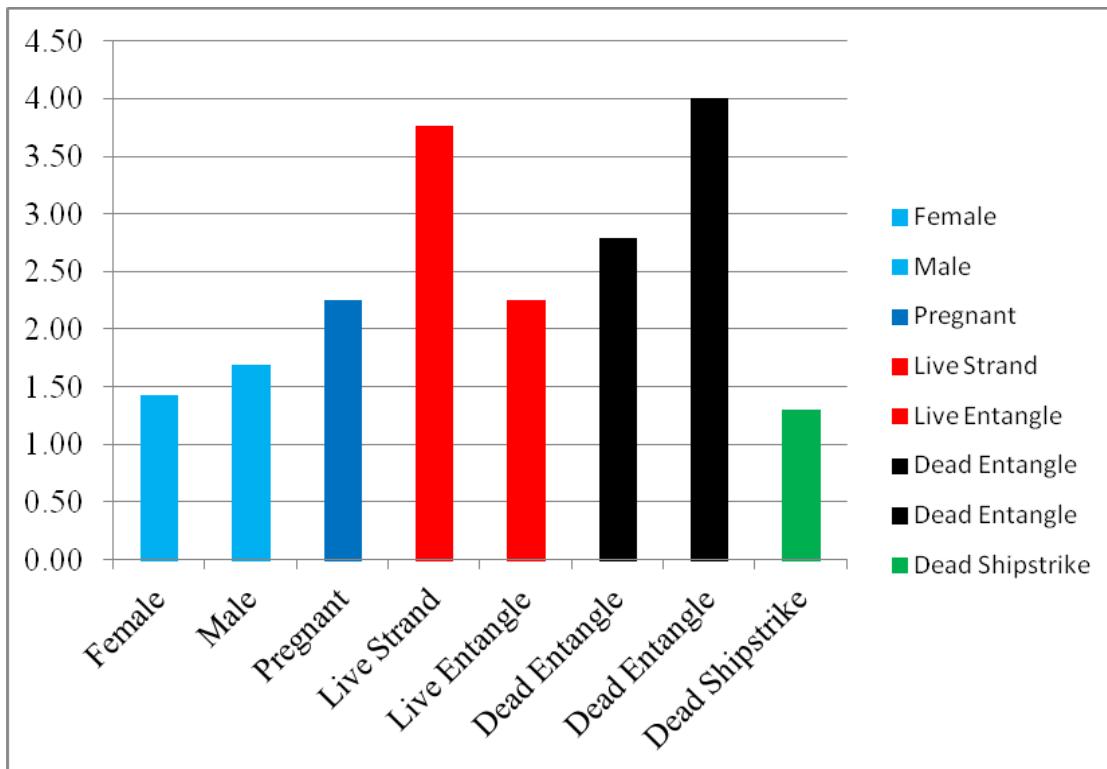


**Figure 2. Change in median fecal glucocorticoid levels from right whales before and after 11 September in the years 2001-2005.**

## TRANSITION 2: PHYSIOLOGICAL/HORMONAL STATUS IS LINKED TO HEALTH, CONDITION, REPRODUCTIVE STATE AND SEASON

### *Linking Hormones and Stressors*

Hormone results from whales subjected to known stressors (e.g. stranding, entanglement) were evaluated for comparison with “normal” whales. For example, right whale #3710 was stranded alive for 3 days, and its GCs were an order of magnitude higher than other living whales, reflecting adrenal cortical activation secondary to extreme physiologic stress (Fig. 3). Thyroid levels were close to the mean for this whale’s age-class ( $56.0 \pm 12.1$  ng/g), showing the lack of thyroid response expected with a short-term stressor. Hormone levels from the stranded whale were compared to mean values in normal males, females, pregnant females, chronic entanglement cases, and 3 whales killed by shipstrike (Fig 3). Pregnant whales showed a normal elevation of GCs related to the metabolic demands of supporting a fetus. The entanglement cases showed elevated GCs related to chronic stress, while whales killed by shipstrike had uniformly low GCs, because sudden death leaves insufficient time for a stress hormone response. Two of three entanglement cases had lower thyroid ( $25.1, 40.6$  ng/g) probably secondary to long-term nutritional stress, while elevated thyroid in the third entanglement ( $129.3$  ng/g) and one shipstrike case ( $115.6$  ng/g), may be due to environmental (e.g. temperature) or other biological factors.



**Figure 3.** *Log<sub>10</sub> fecal glucocorticoids in normal and stressed whales. Males, females, and pregnant females (mean values) are shown, compared to stranded and entangled whales that experienced chronic stress, and whales killed quickly by shipstrike (mean value) that show no evidence of a stress response.*

#### **Linking Body Condition and Stress Levels**

The visual health assessment database was updated for all right whale sightings following Pettis et al. (2004), and now consists of 11,145 batches and 40,794 sightings from 1980-2009. We modeled the relationship between levels of GC hormones and body condition in 80 right whales with fecal hormone data and concurrent body condition scores. No pregnant females had a body condition score other than 1(good condition), and only 1 resting female was available, so they were removed, leaving 64 individuals for analysis. To analyze the relationship between fecal GC levels and body condition, we ran a nested linear model, with GC level as the dependent variable, the other hormone levels nested in reproductive state as the explanatory variables, to which body condition score was added (as a factor), also nested by reproductive state. The change in Akaike Information Criterion (AIC) between the base model and the model including body condition showed significant improvement in the fit of the model to the data, and comparing deviances between the models with an F-test (Venables and Ripley 2002) confirmed this (Table 1). In addition, mature males with body condition greater than 1 (fair or emaciated) had significantly higher GC levels than mature males with body condition=1 (good) ( $t=4.421$ ,  $p<0.001$ ).

**Table 1. Analysis of Deviance Table: Model 1: base, nested model: Model 2: body condition code (nested) added to the model.**

	Residual Df	Residual Deviance	Df	Deviance	F	Pr(>F)
1	44	23746				
2	40	15828	4	7918.9	5.0033	0.002296

### **Validation of Thyroid and Estrogen Hormone Assays in Right Whales**

Thyroid hormones drive metabolic rate in mammals, and tend to decline during long-term stress, potentially providing information on nutritional state and chronic stress (Wasser et al., 2010). We tested both T3 and T4 assays for parallelism. The T4 assay demonstrated no parallelism, and was not tested further. The T3 assay exhibited excellent parallelism ( $F_{2,10}=0.42$ ,  $P=0.6671$ ), and good accuracy. We also tested three new estrogen assays: an estradiol double-antibody RIA, an estradiol coated-tube assay, and an estrone-1-glucuronide EIA (E1G). The E1G assay had excellent parallelism ( $F_{1,10}=0.76$ ,  $P=0.4025$ , ANCOVA), detected high quantities of hormone, and showed strong positive correlations with previous total-estrogens results. This assay produces comparable data, has lower variation, requires less sample, and costs less. The addition of the E1G assay might allow detection of shifts in estrone/estradiol ratios in studies on female reproduction in baleen whales.

### **Characterizing Baseline Hormone Variation by Life History State**

The datasets for the reproductive hormone metabolites (estrogens, progestins, androgens) and adrenal glucocorticoids (GCs) was 370 samples (1999-2011), and 324 for thyroid hormones: the largest database of endocrine measures for any whale species. Of these, 123 samples from identified right whales were used to assess baseline hormone variation with sex, age and reproductive state. Mean hormone levels by life history stage were remarkably consistent with those previously described, demonstrating the robustness of these measures (Hunt et al. 2006; Rolland et al. 2005, 2007).

### **Reproductive Hormones**

The major characteristics were as follows: 1) extreme elevations of progestins in pregnant females, 2) elevated estrogens (without high progestins) in lactating females, 3) mature males with double the testosterone of immature males, and 4) the ratio of fecal androgens to estrogens accurately identified the sex of most whales ( $n = 114$ ; two-tailed  $t = 4.49$ ,  $df = 42.98$ ,  $P < 0.0001$ ).

### **Fecal Glucocorticoids (GC)**

Significant elevations of GCs were seen in pregnant and lactating females, and mature males as described previously ( $n = 114$ ; Hunt et al. 2006). In males, GCs were significantly correlated with age ( $n = 39$ ;  $r = 0.8672$ ,  $P < 0.0001$ ; Hunt et al. 2006). Therefore, **interpretation of GC levels as a biomarker of stress must take into consideration age, sex, and reproductive state of the whale**. Mean annual GCs for 2000-2007 were  $23.9 \pm 0.9$  ng/g (Bay of Fundy and Roseway Basin,  $n = 198$ ), ranging from 15.1 to 36.0 ng/g with significant variability between some years.

### **Thyroid Hormone (triiodothyronine, T3)**

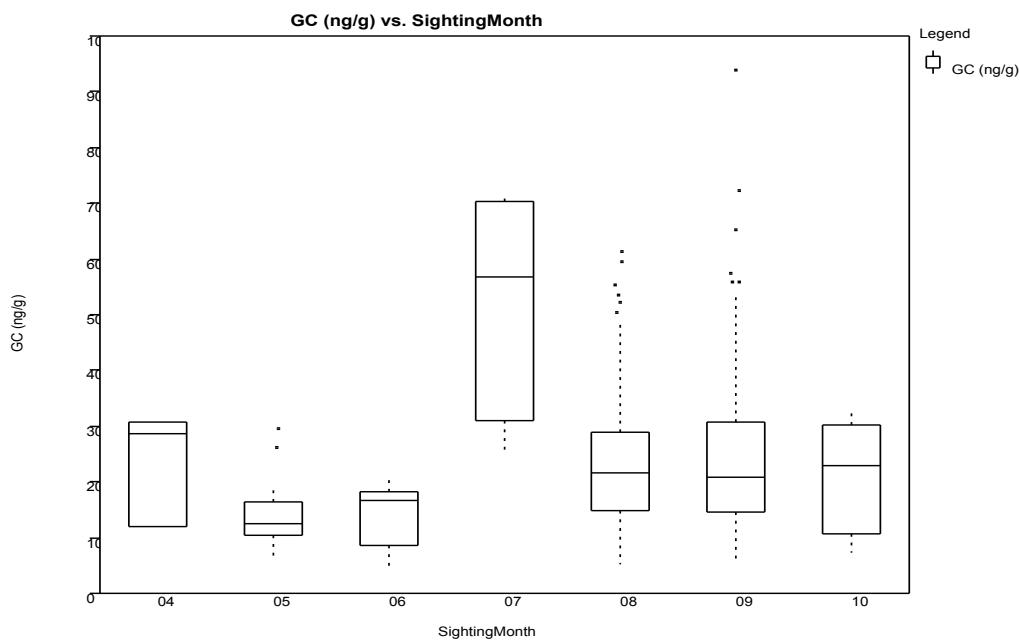
Thyroid hormone showed little variability in whales of different sex and reproductive states ( $50.3 \pm 6.2$  to  $56.0 \pm 12.1$  ng/g;  $n = 97$ ) except for a non-significant trend for lower T3 in resting females ( $36.7 \pm 2.4$  ng/g;  $n = 16$ ). This could be related to an energy-sparing mode in females recovering from the metabolic demands of lactation. There was no correlation of T3 with age (2-31 years) using all samples from known-age whales ( $n = 80$ ). For juveniles (2-8 years;  $n = 28$ ), there was a weak trend for

increasing T3 between 2-5 years of age ( $P = 0.08$ ), likely reflecting the rapid growth occurring during this interval. After age 5, T3 levels appear to level out and remain fairly steady throughout adulthood.

The mean T3 level for all years (from Bay of Fundy and Roseway Basin only) was  $55.2 \pm 2.6$  ng/g ( $n=282$ ). The final data set for the annual comparison ( $n=261$ ) was samples collected in July-October from 2000-2007. Mean T3 varied from  $39.9 \pm 6.3$  to  $79.4 \pm 17.5$  ng/g between years, but the differences were not significant. Mean T3 for all months was  $52.1 \pm 2.4$  ng/g ( $n=306$ ).

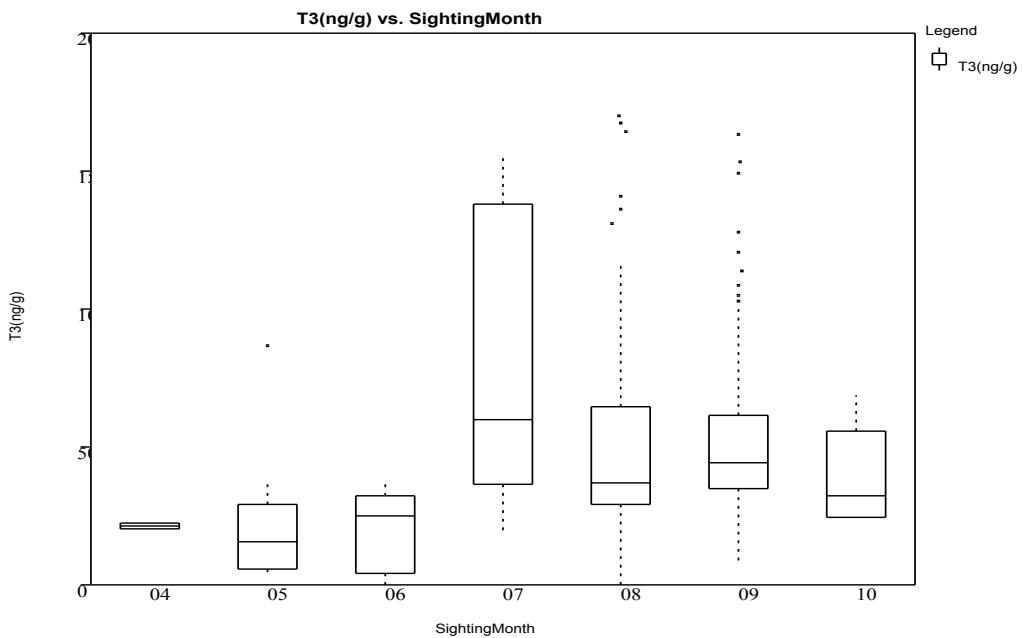
### **Seasonal Variation**

For GCs, there were significant differences between months ( $d.f. = 6,234$ ;  $F = 4.6931$ ;  $P < 0.0002$ ) with May and June well below the mean and July almost double the mean (although based on a very small sample size) (Fig. 4). These monthly differences translate into significant seasonal variation with lower levels in spring ( $n = 24$ ; mean =  $15.5 \pm 1.4$  ng/g) compared to fall months ( $n = 216$ ; mean =  $24.5 \pm 1.0$  ng/g; two-tailed  $t = 4.1159$ ,  $d.f. = 30.016$ ,  $P < 0.0003$ ).



**Figure 4. Fecal glucocorticoids in right whales by month (April to October).**

Mean thyroid levels were compared over 7 months, and T3 was significantly different between months (April-October;  $F=9.7629$ ,  $df=6,295$ ;  $P = 0.0001$ ). Similar to the GCs, there was a pronounced seasonal effect on T3, such that mean T3 in spring was significantly lower than in summer/fall (Fig. 5) (two-tailed  $t = 5.4427$ ,  $df = 22.76$ ;  $P < 0.0001$ ). Lower T3 in spring is likely related to the winter fasting period in which metabolism is down-regulated. **Therefore, there is significant and very similar seasonal variation in both thyroid hormones and GCs.**



**Figure 5. Thyroid hormone (T3) in right whales by month(April to October).**

#### **Relationship between Glucocorticoids and T3**

Glucocorticoids and T3 were correlated with each other ( $n = 352$ ,  $r = 0.375355$ ,  $P < 0.0001$ ), but there is a wide spread to the data, and not a strong correlation. The majority of whales with lower thyroid levels (89%;  $T3 < 25$  ng/g), had normal GCs. Similarly, most whales with higher T3 (60%;  $> 75$  ng/g) had normal GC levels, given the life history state of the whale. Therefore, **these two hormones often vary independently in response to different factors or biological events.**

### **TRANSITION 3: HEALTH AND CONDITION AFFECT VITAL RATES**

#### **Body Condition Effects on Reproduction**

To determine the effects of body condition on reproduction, we analyzed the relationship between body condition and calving in adult females for the period 1980-2009. We used a Markov chain process, which calculates the probabilities of one state transitioning to another, a method used to construct population projection matrices (Caswell 2001). In this analysis we calculated the probability of each adult female right whale transitioning between three possible states – Pregnancy, Lactation and Resting. Only female whales with at least 20 years of sightings as an adult were used in this analysis ( $n=49$ ). None were ever recorded as having a body condition of 3 (emaciated). For each individual whale, a transition matrix was constructed, showing the state that each whale was in each year, along with the stage that the individual moved to in the following year. The popbio library (Stubben and Milligen 2007) was used to construct a projection matrix model from each individual's transition matrix. For all 49 whales, females almost never transitioned from resting with a body condition of 2 (fair condition) to pregnant, indicating that reproduction is contingent on good body condition.

#### **Body Condition Effects on Mortality**

We analyzed body condition (Pettis et al. 2004) and the likelihood that a whale in worse condition dies. The Right Whale Catalog protocol considers an individual whale “dead” if it is not observed for 6

years. As some whales are observed after an interval of 6 years, they become “resurrected”. If body condition influences the likelihood of survival, then the whales that are presumed dead due to a gap in sightings and never seen again (i.e., probably dead) should have worse body condition at their last sighting than those whales that are presumed dead but are later re-sighted. Of 135 whales classified as “dead”, for which visual health assessments were available prior to “death”, 35 were resurrected. We tested whether classifications differed using a Pearson's chi-squared test. As the expected values of some cells were less than 5, we used a Monte Carlo test (10,000 replicates) to calculate a P-value. Proportions differed ( $\chi^2=7.97$ ,  $p<0.05$ ), and residuals showed that whales in fair and poor condition were significantly less likely to be “resurrected” than those in good body condition. Finally, only 11 of 12 right whales last observed with body condition 3 are still alive.

### ***Reproductive and Demographic Classification***

Immunoassays of hormone metabolites from right whale fecal samples provide biologically relevant information on levels of reproductive and stress (GC) hormones (Rolland et al. 2005, Hunt et al. 2006), and responses to anthropogenic stressors (Rolland et al. 2012). To assess whether these measures are reliable indicators of an individual whale's physiologic state, we analyzed all fecal hormone metabolites collected from 102 whales where the gender and reproductive state was known, excluding entangled or ship-struck animals.

Although we initially used Classification Trees (CART) to select which reproductive class a sample belongs to (based on hormone levels), these can lead to splits that are only locally optimal. Here we used a new analytical approach that implements an evolutionary algorithm to search for a globally optimal tree (Grubinger et al. 2011). Reproductive state was the classifying variable, and the five hormones (thyroid, androgens, progesterone, estrogen and glucocorticoids) were the independent variables. We excluded calves, fetuses and juveniles of unknown gender, leaving 96 samples for analysis. Trees were run using the *evtree* library (Grubinger et al. 2011) in R 2.15.1 (R Core Team 2012). Model training used the *caret* library (Kuhn 2012), and processing used the *doMC* library (Revolution Analytics 2012). We partitioned the data into training and test sets, constructed an EVtree model using the training set and tested whether the model provided a good representation of the data using the test set. We reran this process 10,000 times and retained the best model.

This EVTree analysis categorized pregnant females with complete accuracy, mature males, immature males and lactating females with almost complete accuracy, and was less accurate discriminating immature from resting females (Fig. 6). This confirms that hormone metabolite patterns from right whale fecal samples are reliable indicators of the physiologic state of individual whales.

## **RESULTS**

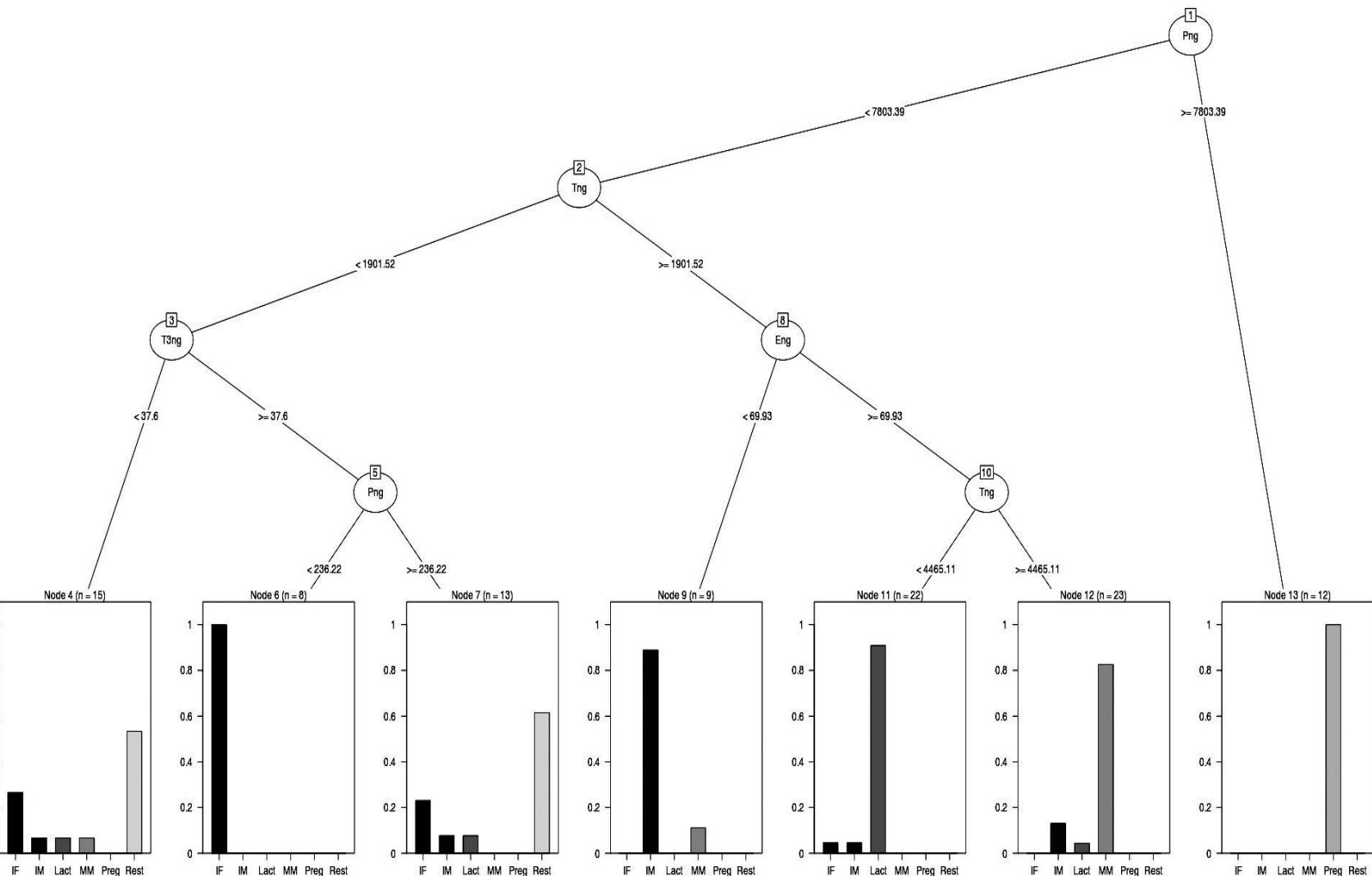
To date we have demonstrated that: 1) acoustic disturbance is associated with elevated GC levels in right whales, 2) higher GC levels are linked with poorer body condition, (3) poor body condition is associated with reduced female reproductive output and increased likelihood of mortality, and (4) reproductive and stress hormone levels can be used to partition demographic and reproductive states in right whales. We have not modeled causation in this approach, but these findings show strong evidence of linkages between underwater noise, stress, body condition (health), and both reproduction and mortality.

## **IMPACT/APPLICATIONS**

The successful use of fecal stress hormones, visual health assessments, and appropriate statistical methods suggest these tools in the appropriate experimental, field, and/or comparative setting, can determine the relationships between anthropogenic (or other) stressors and their effects on whales.

## **RELATED PROJECTS**

As part of the New England Aquarium's Marine Health Program, we have expanded the stress hormone analytical approach to include fecal hormones in beaked and sperm whales (Roz Rolland, PI; ONR Contract # N000141110540), as well as preliminary studies on the detection and use of hormone data from right whale respiratory exudate (Kathleen Hunt, PI; ONR Contract # N000141110435) . In addition, we are currently exploring the application of other stressors (including fishing and health) to the modified PCOD model (Scott Kraus, PI) ONR Contract N000141210389).



**Figure 6. EVTree Analysis of Right Whale Hormone Data. Reproductive states are shown as follows: mature male (MM), immature male (IM), immature female (IF), pregnant female (Preg); lactating female (Lact); and resting female (Rest).**

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Rosalind M. Rolland, Susan E. Parks, Kathleen E. Hunt, Manuel Castellote, Peter J. Corkeron, Douglas P. Nowacek, Samuel K. Wasser and Scott D. Kraus. 2012. Evidence that ship noise increases stress in right whales. Proc. R. Soc. B 279:2363-2368.